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Metabolic cooperation between mouse embryonal carcinoma cells and their differentiated derivatives

(embryonic development/*in vitro* differentiation/cellular interaction/gap junctions/human teratocarcinoma)

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ABSTRACT **Metabolic cooperation has been used as an index of intercellular communication between mouse embryonal carcinoma (EC) and other cell types. EC cells do not cooperate with differentiated cells of various properties or origins. In contrast, they cooperate with cells of all other EC lines tested, including a human teratocarcinoma line, but with different efficiencies. This might reflect differences either in cell–cell interactions or in the formation of gap junctions. During *in vitro* differentiation, EC cells appear to remain isolated from differentiated cell types.**

Although communication between embryonic cells by means of junctions is often assumed to be involved in development (1), little is known as yet about its precise role. Gap junctions between blastomers have been shown to appear at the 8-cell stage, followed by tight junctions and desmosomes. The last two structures probably contribute to the formation of the blastocoele (2, 3).

Intracellular communication can be studied by using the teratocarcinoma as a model system of differentiation (4). From this material, multipotential embryonal carcinoma (EC) cell lines have been established which resemble the multipotential cells of the embryo (5, 6). Differentiated cell lines, some of which correspond to early stages of differentiation in the embryo, have also been established. Finally, some EC cell lines are able to differentiate *in vitro*, either spontaneously or after treatment with some chemicals (7). This system therefore allows study of early differentiation of multipotential cells (8).

Metabolic cooperation (9) has been used to demonstrate transfer of molecules between cells by autoradiography. The cells of an EC line have been shown to become metabolically coupled with hamster fibroblasts (10). This paper reports the use of another technique to study metabolic cooperation of EC cells. With this technique, it is possible to examine easily cooperation between many different cell lines and to quantify the results.

MATERIALS AND METHODS

Cells and Culture Conditions. The origin and nature of the cell lines used are summarized in the tables. The Don hamster fibroblast cell line was obtained from Gérard Buttin and the F4Cl III N cell line was from Harvey Eisen. Fibroblasts in primary culture were derived from the skin of 129/Sv mice. LT/AzaR1, PCC3/AzaR1, and PCC4/AzaR1 cell lines were selected in a medium containing 15 μg of azaguanine per ml and shown to be deprived of hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) (L. Soriano, personal communication). All cells were cultivated in Eagle's medium (Dulbecco's modification) supplemented with 15% fetal calf serum. The cultures were grown in a humidified incubator in an air/12% CO_2 mixture at 37° on Falcon tissue culture dishes as described (8). All cell lines were periodically checked for the absence of mycoplasma.

Tumors. These were obtained by injecting 2–5 × 10^6 cells into syngeneic mice (irradiated with 650 rads or not) as described (8). EC cells were considered to be nullipotent when no differentiation was observed in more than 20 independent tumors. They were considered to be multipotent when derivatives of the three germ layers were observed in the tumors.

Hybrid Cells. Hybrid clones were obtained by fusion of two cell types with polyethylene glycol. Selection was done in a medium containing hypoxanthine, aminopterin, and thymidine (HAT), when the parental cells were respectively $HPRT^-$ and thymidine kinase negative (cross B) or in HAT medium containing 1 mM ouabain when one parent cell was wild type and the other was a double mutant $HPRT^-$, ouabain-resistant (OR) (crosses A and C). In all cases, one of the parents was a multipotential EC cell and the other was a differentiated cell. The hybrid nature of the clones was ascertained by the number of chromosomes and the presence of metacentric marker chromosomes (crosses A and B). In cross C, in which the parental cells differ by alleles at the glucosephosphate isomerase locus, both products were shown to be present in the hybrid.

***In vitro* Differentiation.** *Confluence differentiation* (11). PCC3/A/1 cells (1.5 × 10^6) were plated on 100-mm tissue culture dishes in Eagle's medium containing 15% fetal calf serum. The cells were allowed to differentiate for 14 and 28 days, the medium being changed every 2–3 days. After differentiation, the cells were dissociated with a solution of trypsin in phosphate-buffered saline, replated (3 × 10^6 cells per 100-mm culture dish) to allow multiplication, and tested 2 days later.

Induced differentiation (7). PCC3/A/1 cells (3 × 10^6) were plated on 100-mm tissue culture dishes in a medium containing 5 mM hexamethylene bisacetamide; the medium was renewed every 2–3 days. The cells were used after 6 days of treatment.

Measure of Metabolic Cooperation. In HAT medium, $HPRT^-$ cells die (because of a lack of HPRT and the presence of aminopterin), whereas $HPRT^+$ cells multiply normally. When both cell types are grown together under certain conditions, $HPRT^+$ cells can rescue the $HPRT^-$ ones by transfer of metabolites (metabolic cooperation). $HPRT^-$ EC cells are very sensitive to HAT medium, in which they lyse. If such cells are labeled with [^{14}C]thymidine, the amount of lysis can be measured by the fraction of radioactivity released into the

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Abbreviations: EC, embryonal carcinoma; HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); HAT, hypoxanthine/aminopterin/thymidine; AzaR, azaguanine-resistant; OR, ouabain-resistant.

medium. Metabolic cooperation can thus be determined by measuring the decrease of radioactivity released from mixed populations of radiolabeled HPRT$^-$ and nonlabeled HPRT$^+$. For these experiments, 5×10^6 HPRT$^-$ cells were incubated for 12 hr in the presence of 2 μCi of [2-^{14}C]thymidine (53 mCi/mmol). The radioactive medium was then replaced by unlabeled medium. Two hours later, the cells were dissociated with phosphate-buffered saline, washed in Eagle's medium, and replated in tissue culture wells (Costar). HPRT$^+$ cells were dissociated and then added to the wells. The cells were allowed to attach for 4 hr. In half the wells, the medium was then replaced with HAT medium. Controls without HPRT$^+$ cells were grown in parallel.

To a constant number (generally 2×10^5) HPRT$^-$ cells, a series of 1:2 dilutions (ranging from 6×10^5 to 10^4) of HPRT$^+$ cells was added. Two days later, the cultures were observed with an inverted microscope to detect the presence of HPRT$^-$ cells, when a difference in morphology between the two cells made this possible. The supernatant of each well and the cell debris (harvested with 0.5 ml of phosphate-buffered saline) were collected. Viable cells were detached with trypsin. Radioactivity was measured in both supernatant and cells, by using 10 ml of Unisolve (Koch-Light Lab) as scintillator liquid. Radioactivity was assayed in an Intertechnique SL40 spectrometer counter. The fraction of *released* radioactivity (a) due to HAT medium was determined according to the following calculation: a = (% radioactivity in HAT supernatant − % radioactivity in normal supernatant)/(100 − % radioactivity in normal supernatant). This value was then normalized to the *releasable* fraction of radioactivity (b) determined with the same formula as a, from wells to which no HPRT$^+$ cells were added. Finally, a/b values (lytic index) were plotted against the logarithm of the ratio HPRT$^+$/HPRT$^-$ cells. A lytic index of 1 corresponds to complete lysis of HPRT$^-$ cells.

RESULTS

In HAT Selective Medium, HPRT$^-$ EC Cells Are Protected from Lysis by HPRT$^+$ EC Cells. A constant number of [^{14}C]thymidine labeled HPRT$^-$ cells were plated with an increasing number of nonradioactive HPRT$^+$ cells. The lysis of [^{14}C]thymidine-labeled HPRT$^-$ cells was estimated by measuring the radioactivity present in the HAT supernatant (Fig. 1). In the case of PCC4/AzaR1 and PCC3/A/1 cells, a decrease of lysis was observed when the number of HPRT$^+$ cells increased. To protect 80% of 2×10^5 HPRT$^-$ cells (lytic index = 0.2), 2×10^5 HPRT$^+$ cells were needed. Below this number, the percentage of radioactivity released by lysis increased linearly with the logarithm of the number of HPRT$^+$ cells and reached 85% when the number of HPRT$^+$ cells was equal to or less than 0.2×10^5. The ratio (number of HPRT$^+$ cells/number of HPRT$^-$ cells) required for total protection of HPRT$^-$ cells was obtained by extrapolating the linear portion of the curve to the abscissa. For PCC4/AzaR1 and PCC3/A/1, this ratio was 0.9.

To exclude that in such experiments the decrease of radioactivity in the supernatant might result from its reutilization by HPRT$^+$ cells, the following experiments were done. First, irradiated, radiolabeled HPRT$^-$ cells were added to nonlabeled HPRT$^+$ cells. Irradiation induces cell lysis with kinetics similar to those observed in HAT medium. Even in the presence of a large number (6×10^5) of HPRT$^+$ cells, all the releasable radioactivity remained in the supernatant (Fig. 1; Table 1). Second, when the ratio HPRT$^+$/HPRT$^-$ was equal to or greater than 0.5, the total cell number in both normal and selective medium remained approximately the same, a result that also supports the conclusion that HPRT$^+$ cells protect the HPRT$^-$ variants from lysis.

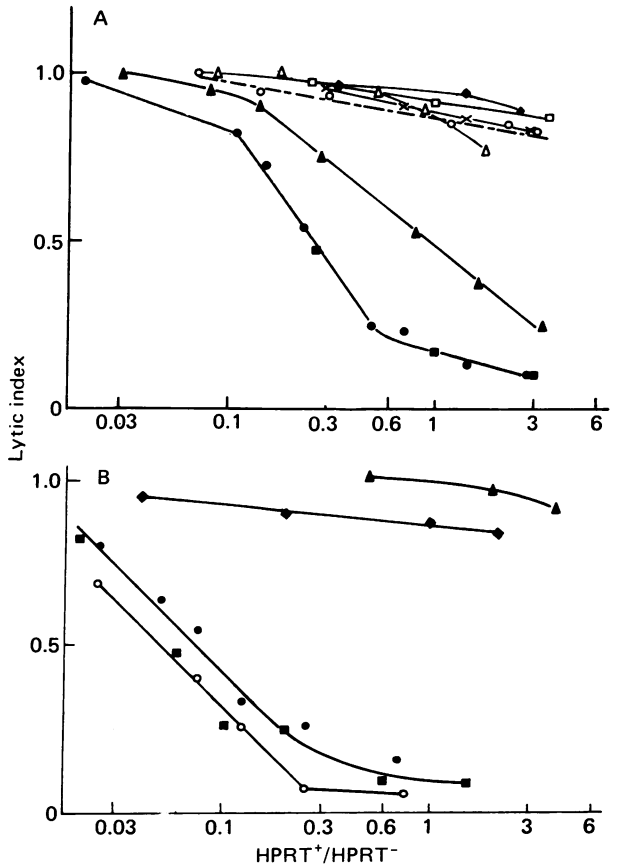

FIG. 1. Metabolic cooperation between various HPRT$^+$ cell lines and PCC4/AzaR1. (*A*) 2×10^5 PCC4/AzaR1 cells were mixed with increasing numbers of PCC3/A/1 (●, ■, two independent experiments), F9 (▲), 3/TDM1 (△), *in vitro* differentiated PCC3/A/1 at day 14 (♦), hexamethylene bisacetamide-differentiated population of PCC3/A/1 (x), or primary culture of mouse fibroblasts (□). Control, irradiated (1800 rads) PCC4/AzaR1 (○--○). (*B*) 2×10^5 PCC4/AzaR1 cells were mixed with increasing numbers of hybrid cell lines: (P × F) (EC phenotype) (○), (P × C) (non-EC phenotype) (♦), and the parental lines PCC4 (●, ■, two independent experiments) and F4Cl III N (▲).

Protection of HPRT$^-$ Cells by HPRT$^+$ Cells in Selective Medium Needs Close Contact between Cells. If the decrease of radioactivity does indeed depend on contact between HPRT$^-$ and HPRT$^+$ cells, this effect should vary with the possibility for the two cell types to establish contact (i.e., with cell density). This was studied by plating cells in a constant ratio (HPRT$^+$/HPRT$^-$ = 3), but at different densities, in selective medium (Fig. 2). The protection decreased rapidly when the total cell number per well was smaller than 2×10^5. Another approach consisted in plating groups of 10^4 cells, either dissociated or aggregated, with a constant number of HPRT$^-$ cells (Fig. 3). (When the HPRT$^+$ aggregates are dissociated the probability of contact with HPRT$^-$ cells is maximal; it is greatly decreased when HPRT$^+$ cells remain as clumps.) Only in the former case was protection observed. Finally, when coverslips on which PCC4/AzaR1 cells were grown were placed in dishes containing PCC3/A/1 cells in HAT medium, complete lysis of HPRT$^-$ cells occurred in 2 days. These results suggest that cell-to-cell contact is indeed required for protection to occur, the supernatant playing no role in the phenomenon.

EC Cells Cooperate with All Other EC Cell Types. The types of cells able to rescue EC cells were investigated by mixing

Table 1. Metabolic cooperation between PCC4 AzaR1 and differentiated cells

Cell type	Cell line	Malig-nancy*	Lytic index† HAT medium	Lytic index† Irradiated control	Origin (mouse)	Ref.
EC, multipotent	PCC4	+	0.05	0.73	129/Sv	(12, 11)
EC, nullipotent	LT1	+	0.12	0.88	LT	(13)
Trophoblastoma	3/TDM1	+	0.68	0.60	129/Sv	(8)
Parietal endoderm	PYS-2	+	0.69	0.69	129/Sv	(14)
Osteogenic mesenchyme	3/A/1-D-1	−	0.92	ND	129/Sv	(Unpublished)
Embryonal fibroblast	3/A/1-D-3	−	0.96	ND	129/Sv	(Unpublished)
Preadipocyte	3/A/1-D-4	−	0.90	ND	129/Sv	(Unpublished)
Embryonal fibroblast	3/A/1-D-5	+	0.95	0.84	129/Sv	(Unpublished)
Adult fibroblast	Primary culture	−	0.82	0.84	129/Sv	
Myoblastic cells	C17-S1-D-T984	−	0.90	ND	C3H	(15)
Erythroleukemic cells	F4Cl III N	+	0.92	ND	DBA/2	
Fibroblast	Don	−	0.78	0.65	Chinese hamster	
In vitro differentiated PCC3/A/1	Day 7‡	−	0.88	0.95		
	Day 14§	−	0.87	0.70	129/Sv	(7, 11)
	Day 28§	−	0.65	0.62		

* Determined by injection of 2–5 × 10^6 cells into syngeneic mice. +, Malignant; −, nonmalignant.
† $HPRT^+/HPRT^-$ ratio = 3. ND, not determined.
‡ Induced with hexamethylene bisacetamide.
§ Differentiation at confluency.

LT/AzaR1 (a nullipotent cell line) or PCC4/AzaR1 or PCC3/AzaR (two multipotent cell lines) with various cells. The results are given in Tables 1 and 2. Two classes of cells could be distinguished: (*i*) those with which the lytic index a/b in HAT medium does not decrease with an increasing number of $HPRT^+$ cells; and (*ii*) those with which the lytic index decreases with an increasing number of $HPRT^+$ cells. Only in the latter case does metabolic cooperation occur.

Whatever their properties, morphology, or origin, all EC cell lines tested cooperated with the three EC tester lines (Table 2). However, there was a large variation in the minimum number of cells required to protect the same number of $HPRT^-$ cells. On this basis, it is possible to classify EC cell lines and two conclusions can be drawn: (*i*) for protection to occur, a given cell line requires fewer cells of the same line than of other EC cell lines, and (*ii*) the different EC lines do not exhibit the same efficiency of protection toward LT/AzaR1 and PCC4/AzaR1 cells.

Special mention should be made of the cooperation between PCC4/AzaR1 and two cell lines derived from human teratocarcinomas Tera I and Tera II (19). These two lines differ in that Tera II carries the embryonic antigen F9 (5) whereas Tera I expresses no F9 antigen and exhibits an endodermal-like morphology. Only the cell line that bears the F9 antigen, Tera II, was found to cooperate.

EC Cells Do Not Cooperate with Differentiated Cells Derived from Teratocarcinoma or from Adult Mice. A number of differentiated cells were tested for an eventual

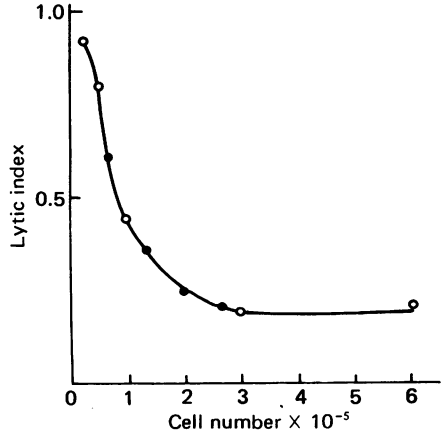

FIG. 2. Effect of cell density on metabolic cooperation. Increasing amounts of the same mixture ([^{14}C]thymidine-labeled PCC3/AzaR cells and PCC3/A/1 cells in a ratio 3:1) were plated in wells and the lytic index was determined 48 hr later. ○ and ●, Two independent experiments.

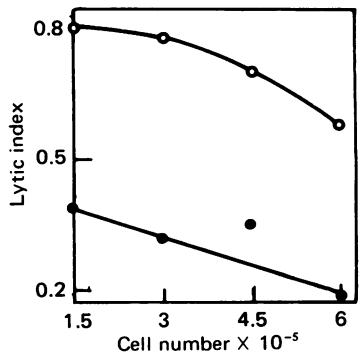

FIG. 3. Effect of cell dissociation on metabolic cooperation. PCC3/A/1 cells were incubated for 1 day in Petri dishes in which they formed aggregates. Increasing amounts of this suspension were plated, either as aggregates (○) or after cell dissociation (●), with 2 × 10^5 dissociated PCC4/AzaR1 cells labeled with [^{14}C]thymidine. The lytic index was determined 48 hr later. Abscissa: number of PCC3/A/1 cells.

Table 2. Metabolic cooperation between EC cells

Cell type	HPRT+ cell line	No. HPRT+ cells ($\times 10^{-5}$) required to rescue 2×10^5 HPRT−* PCC4/AzaR1	PCC3/AzaR	LT/AzaR	Origin (mouse)	Ref.
EC, multipotent	PCC4	0.54	6	6.6	129/Sv	(12, 11)
EC, multipotent	PCC3/A/1	2	4	6.6	129/Sv	(8)
EC, nullipotent	LT1	6	22	1.8	LT	(13)
EC, nullipotent	F9	20	50	10	129/Sv	(5)
EC, multipotent	PCC3/A/1-D-G2	1.5	NT	20	129/Sv	(16)
EC, multipotent	PCC7-S	26	NT	70	(129 × B6)	(17)
EC, multipotent	C17-S1/A	4.6	NT	5.6	C3H	(18)
Human teratocarcinoma-derived cell lines	Tera I	No coop.†	NT	NT	Human	(19)
	Tera II	3.4	NT	NT	Human	(19)

* Determined by extrapolating curves similar to those in Fig. 1. NT, not tested.
† No cooperation.

metabolic cooperation with EC cells. These included populations of PCC3/A/1 at different stages of *in vitro* differentiation and populations of PCC3/A/1 induced to differentiate by hexamethylene bisacetamide. In addition, a series of established lines was used corresponding to early or late stages of differentiation: 3/TDM1, which gives rise to giant trophoblastic cells and has the characteristics of a trophoblastoma; PYS-2, which secretes material crossreacting with Reichert's membrane of the embryo and corresponds to parietal endoderm; 3/A/1-D-1, which gives rise to nonmalignant bone and probably corresponds to a mesenchymal cell line; and 3/A/1-D-3, which corresponds probably to a primitive form of fibroblast. In no case was metabolic cooperation observed either with LT/AzaR1 or with PCC4/AzaR1 (Table 1). The small decrease of radioactivity in the supernatant observed with *in vitro* differentiated populations of PCC3/A/1 is probably due to some reabsorption by certain differentiated cells, as suggested by the irradiated control.

Finally, none of the cells in a more advanced state of differentiation or derived from the adult mouse (Table 1) was able to cooperate with EC cells, although some of these cells, such as primary mouse fibroblasts and Don hamster fibroblasts, are known to cooperate with some other cell lines (9).

EC Cells Cooperate Only with Those Hybrid Cells that Have Characteristics Similar to those of EC Cells. Hybrids formed by fusion between EC and differentiated cells are of two types: some have properties similar to those of the EC parent (presence of F9 antigen and ability to give rise to differentiated tumors); others have different properties (21). The capacity for rescuing EC cells in HAT medium was therefore investigated with both hybrid types. Only those hybrids that had an EC phenotype could rescue HPRT− EC cells in HAT medium (Table 3).

DISCUSSION

HPRT+ cells protect HPRT− cells from lysis in HAT medium. The amount of protection can be used as a measure of metabolic cooperation. The method is quantitative and allows comparison between cell lines. Its sensitivity depends primarily on the

Table 3. Metabolic cooperation between EC cells and hybrids (EC × differentiated cells)

Cross		Cell lines	Cell type and ref.	Presence of F9 antigen*	Metabolic cooperation with EC cells†
A	Parents	PCC4/AzaR-OR	EC, multipotent (8)	+	+ (0.50×10^5)
		F4C1 III N	Friend erythroleukemia	−	−
	Hybrid	(P × F)	EC, multipotent‡	+	+ (0.72×10^5)
B	Parents	PCC4/AzaR1	EC, multipotent (8)	+	+ (0.54×10^5)
		LC28F ($BudR^R$)	Lymphoma (20)	−	Not tested
	Hybrids	(P × L) 1	EC, multipotent‡	+	+ (5.0×10^5)
		(P × L) 2	Non-EC‡	−	−
C	Parents	PCC3/AzaR-OR	EC, multipotent (8)	+	+ (2.2×10^5)
		C17-S1-D-T984	Myoblasts (19)	−	−
	Hybrid	(P × C)	Non-EC‡	−	−

* Detected by absorption with a 129 Sv anti-F9 antiserum, followed by cytotoxicity test against F9 cells (5). +, Presence; −, absence.
† HPRT+ cells were tested with PCC4/AzaR1; HPRT− cells were tested with PCC4. Numbers in parentheses indicate how many cells were required to rescue 2×10^5 HPRT− cells. +, Cooperation; −, no cooperation.
‡ Unpublished.

sensitivity of the cells to aminopterin as compared with the rate of adhesion between cells and with the amount of metabolites transferred between cells. Although probably not as sensitive as autoradiography, this method allows one to study a large number of cell lines, even when their morphologic features are identical.

EC cells cooperate between themselves but not with differentiated cell types, whatever their state of differentiation or origin. The latter result is not in agreement with a previous report (10) indicating that PC18 EC cells and Don hamster fibroblasts can cooperate. This discrepancy may be due to a difference in the EC line used or to a difference in the sensitivity of the two techniques.

In our experiments, it is unlikely that the inability of differentiated cells to cooperate with EC cells is due to a difference in metabolism between EC cells and all other cell types because all the azaguanine-sensitive cell lines used have HPRT activity and are equally sensitive to azaguanine or 6-thioguanine. In addition, differentiated cells derived from PCC3/A/1 AzaR are equally HAT-sensitive. It is also unlikely that the inability of differentiated cells to cooperate with EC cells is due to a lack of physical contact, because $HPRT^-$ EC cells plated on a differentiated cell monolayer are not rescued, even when the selective medium is added 24 hr after plating.

The most likely explanation for the cooperation between EC cells is the formation of gap junctions which are known to be involved in electric coupling and transfer of molecules (22). EC cells have been shown to establish gap junctions (I. Dunia and E. L. Benedetti, personal communication). The lack of metabolic cooperation between EC cells and differentiated cells might be due to a lack of gap junctions between such cells, although Don cells (22), fibroblasts, and 3/A/1-D-1 (I. Dunia and E. L. Benedetti) are known to establish gap junctions. This would imply the existence of a specificity during the formation of gap junctions, perhaps at the cell–cell recognition level. Although rare, such a specificity has already been described in certain systems (23, 24).

EC cell lines are known to differ from each other by certain properties such as the capacity to differentiate and surface antigens (25). Cooperation between nullipotential cells LT and LT/AzaR is as efficient as that between multipotential PCC3 and PCC3/AzaR. Thus, the nullipotentiality of this cell line cannot be ascribed to a lack of intercellular communication. Whatever their origin, all EC lines cooperate between themselves but to a different extent. Further work is needed to establish some correlation between these different properties of EC cells. However, the fact that mouse EC lines do cooperate with a human teratocarcinoma line implies that the surface structures involved have been conserved during evolution. It is of interest to note that human EC cell lines have also conserved F9 antigen (19, 26). The fact that EC cells cooperate between themselves, but not with differentiated cells, indicates that groups of cells can remain isolated from differentiated cells as differentiation proceeds.

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